AMENDMENTS TO THE DRAWINGS

Replacement drawing sheets with FIGURES 4-7 are attached herewith. In replacement FIGURES 4-7, the lanes have been labeled.

<center>REMARKS</center>

Claims 58-97 are pending. Claims 59 and 76-97 have been withdrawn from consideration as directed to non-elected inventions. Claims 58 and 60-75 have been rejected. Claims 61, 63, 66, and 71 have been objected to. Claims 58 and 60-75 have been amended. Reconsideration and allowance of Claims 58 and 60-75 is respectfully requested in view of the above amendments and following remarks.

<center>Amendments to the Specification</center>

Title. The title has been objected to as not descriptive of the elected invention. The title has been amended. Withdrawal of the objection is respectfully requested.

Drawings. Figures 4-7 have been objected to because the lanes are not labeled or clearly described. Replacement figures containing the appropriate labels are submitted. Withdrawal of the objection is respectfully requested.

Abstract. The abstract has been objected to. A new abstract is submitted herewith.

Arrangement of the Specification. The specification is objected to for improper formatting. The specification has been amended to include section headings. Withdrawal of the objections to the specification formatting is respectfully requested.

<center>Objection to Claims</center>

Claims 61 and 63. Claims 61 and 63 have been objected to for reciting non-elected subject matter, specifically conjugates comprising a DNA sequence. Claims 61 and 63 have been amended to remove all reference to DNA sequences. In view of the amendments, withdrawal of the objection is respectfully requested.

Claim 66. Claim 66 has been objected to for an improper comma. Claim 66 has been amended. In view of the amendment, withdrawal of the objection is respectfully requested.

MASQ27218AM2.DOC

Claim 71. Claim 71 has been objected to for being redundant with Claim 69. Additionally, the Office Action states that it is assumed that Claim 71 is meant to depend from Claim 70. Claim 71 has been amended to depend from Claim 70. However, Applicants respectfully traverse the objection that Claim 71 is redundant to Claim 69. In this regard, Claim 69 states, in part, that "said detecting is performed by . . . fluorescent-ligand binding techniques, or radio-ligand binding techniques," whereas Claim 70 does not. Additionally, Claim 70 states, in part, that "said detecting is performed by . . . intra- and extracellular assessment of transported material," whereas Claim 69 does not. Because wording exists in each claim that is absent from the other, applicants submit that Claims 69 and 71 are not redundant. Withdrawal of the objection is respectfully requested.

The Obviousness-Type Double Patenting Rejection of Claims 58, 60-63, 74

Claims 58, 60-63, 74, and 75 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claim 33 of U.S. Application 11/791,979. The Office Action asserts that the claims are not patentably distinct from each other. In this regard, the Office Action concedes that the claims differ in that Claim 33 of U.S. Application 11/791,979 recites the limitation of methods for making a conjugate that inhibits exocytic fusion in a nociceptive sensory afferent cell, wherein the claims of the present invention do not. However, the Office Action asserts that the portion of the specification in U.S. Application 11/791,979 that supports Claim 33 includes embodiments that would anticipate or fall within the scope of the present claims.

Applicants note that, according to MPEP § 804(B)(1), if a provisional obviousness-type double patenting rejection is the only remaining rejection against an earlier-filed application, the rejection should be withdrawn and addressed in the later-filed application. The present

application was filed on September 13, 2004, with a priority date of September 11, 2003. U.S. Application 11/791,979 has a later priority date, December 1, 2004.

The Rejection of Claims 58 and 60-75 Under 35 U.S.C. § 112, Second Paragraph

Claims 58 and 60-75 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. The claims have been amended to address the points raised by the Office Action.

Claim 58 has been amended to recite "A method of preparing . . . " support for the amendment can be found at page 4, lines 38-39 of the application.

Claim 60 has been amended to remove the language objected to by the Examiner.

Claims 61 and 63 have been amended to remove all reference to DNA sequences. Claim 61 has been further amended to remove the phrase "an agent of the present invention".

Claims 69, 71, and 73 have been amended to depend from Claims 68, 70, and 72.

Claims 60-75 have been amended to correct antecedent basis.

In view of the amendments, withdrawal of the rejection is respectfully requested.

The Rejection of Claims 58 and 60-75 Under 35 U.S.C. § 112, First Paragraph

Claims 58 and 60-75 stand rejected under 35 U.S.C. § 112, first paragraph, for failing to enable the full scope of the claims. The Office Action states that the specification is enabling for making a conjugate comprising the IL-13 targeting agonist and the catalytic and translocation domains of IgA protease. However, the Office Action asserts that the enablement provided by the specification is not commensurate with the scope of the claims. In this regard, the Office Action dismisses several examples for making conjugates because "the structures/identities of said fragments have not been disclosed." Applicants respectfully submit that the specification enables the full scope of the claims for the following reasons.

Applicants respectfully submit that the present specification provides enabling description for the preparation of the claimed conjugates. The amount of guidance or direction

MASQ\27218AM2.DOC

needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). Furthermore, a patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

The present specification exemplifies the preparation of conjugates having a range of targeting moiety, protease, and translocation domains, well beyond that of IL-13 targeting agonist and the catalytic and targeting moiety domains described in Example 8. Specifically, Example 3 discloses the preparation of an IL-13 - LH_N/C conjugate, Example 11 discloses the preparation of an Insulin - LH_N/B conjugate, Example 15 discloses the preparation of a MCD peptide - LH_N/C conjugate, Example 19 discloses the preparation of an IL-4 - LH_N/C conjugate, Example 22 discloses the preparation of a TNFα - LH_N/C conjugate, and Example 25 discloses the preparation of an EGF - LH_N/C conjugate. The structures of these fragments have been extensively characterized in the art (see, e.g., page 1, line 37 to page 3, line 37 of the present specification, and page 12, line 13 to page 15, line 22).. Thus, the structures of these fragments and the expected tolerance of specific amino acids to modification would be well-known to the skilled person in the art. Specific disclosure thereof is not required to be enabling to the skilled person. Therefore, applicants respectfully submit that these examples provide enabling disclosure supporting the claimed invention.

In addition to these examples, a skilled person, without undue experimentation, would be able to identify alternative targeting moieties, translocation domains, and proteases to generate a conjugate according to the invention for the following reasons.

<u>Targeting moieties / Agonists</u>. The skilled person would be familiar with a number of entirely conventional methods suitable for identifying molecules having the desired activity. As recited in the specification at page 6, lines 6-7, an agonist can be any molecule that is capable of increasing the concentration of exocytic fusion in a target cell (e.g., by increasing secretion or by increasing the concentration of cellular membrane proteins such as receptors, transporters, or membrane channel proteins.) No undue burden is required to carry out a review of the available art literature, as evidenced by the Examiner's own routine search, which has identified documents describing agonists such as insulin (though not in the context of re-targeted toxin conjugates).

In addition, as discussed on page 6, lines 13-17, methods suitable for identifying molecules having the desired agonist activity also include any method of directly or indirectly measuring cellular secretion or the concentration of a membrane protein in a target cell. Conventional methods for measuring secretion are provided in the present specification on page 6, lines 19-23. Conventional methods for identifying a change in concentration or density of a cell membrane protein are discussed in the passage spanning page 6, line 25, to page 7, line 2. A number of these assays are illustrated in the Examples of the present specification: Example 1 (ELISA); Example 9 (immunofluorescence and liquid scintillation counting); Example 18 (flow cytometry); Example 23 (electrophysiology); and Example 26 (Western blotting).

The effectiveness of the candidate agonist molecule may be confirmed by *in vitro* and / or *in vivo* assays (see, e.g., page 7, lines 13-17, page 10, lines 7-20, and Examples 1, 9, 14, 18, 21, 23 and 27 of the present specification). Further details of suitable assays for the desired agonist activity are provided on pages 10-11 of the present specification.

Thus, in the light of the present specification, the skilled person would, without undue experimentation, identify a targeting moiety / agonist suitable for use in the present invention.

MASQ27218AM2.DOC

Thus, the targeting moiety / agonists recited in the pending claims are fully enabled by the specification as filed.

  <u>Proteases</u>. Proteases suitable for use in the present invention are (i) non-cytotoxic (i.e., they do not kill the cell to which they are targeted, see, e.g., page 12, lines 13-15) and (ii) capable of cleaving one or more proteins of the exocytic fusion apparatus in a target cell. The skilled person, without undue experimentation, would be able to identify a protease (or fragment) having these desired activities. In this regard, "non-cytotoxic" activity can be assayed for using an entirely straightforward measure of cell viability after exposure to the protease. Whether a molecule is capable of cleaving a protein of the exocytic fusion apparatus can be assayed using the conventional method described at page 11, lines 22-28, of the present specification (detection of cleavage of a substrate (SNARE protein) by monitoring disappearance of substrate or appearance of cleavage product, for example, by using an antibody).

  The specification also provides numerous specific examples of proteases suitable for use in the claimed conjugates. Preferred proteases include bacterial proteases (or fragments thereof), for example, a clostridial L-chain or a neisserial IgA protease (e.g., from N. gonorrheae). Preferably, a protease for use in the presently claimed conjugates has serine or metalloprotease activity, preferably specific for a SNARE protein such as SNAP-25, synaptobrevin, or syntaxin (see page 12, lines 17-28). Particularly preferred proteases are derived from neurotoxins, especially bacterial neurotoxins such as clostridial neurotoxins, as described in detail in the present specification (page 1, line 24 to page 2, line 22, and page 12, line 35 to page 13, line 3).

  Thus, in light of the present specification, a skilled person, without undue experimentation, would be able to identify a non-cytotoxic protease suitable for use in the present invention. Thus, the non-cytotoxic proteases recited in the pending claims are fully enabled by the specification as filed.

MASQ\27218AM2.DOC

Translocation domains. The translocation domains of the presently claimed conjugates translocate the protease component (above) from within the endosome, across the endosomal membrane and into the cytosol of the target cell. A skilled person would be familiar with a number of conventional assays for determining whether a given molecule possess the required translocation activity, as discussed on page 13, lines 5-25 of the present specification.

In addition, a skilled person would be aware of a number of bacterial and viral sources of suitable translocation domains, including translocating domains of enzymes such as bacterial toxins. As discussed on page 2, lines 1-9, and pages 13-14 of the present specification, the translocation activity of the HN domain of clostridial neurotoxins (Botulinum types A, B, C, D, E, F and G neurotoxin, and Tetanus neurotoxin) has been well documented prior to the present application. Thus, clostridial neurotoxin HN domain is useful as a translocation domain in the presently claimed conjugates.

Non-clostridial translocation domains were also known prior to the present application, and are useful in the conjugates of the presently claimed invention. A number of specific examples are given on pages 14-16 of the present specification (in particular, Table 1).

Thus, in light of the present specification, a skilled person, without undue experimentation, would be able to identify a translocation domain suitable for use in the present invention. Thus, the translocation domains recited in the pending claims are fully enabled by the specification as filed.

Agonist-protease-translocation domain conjugates. A skilled person would also face no undue burden in assembling the above-described individual components to generate a conjugate as recited in the pending claims. Furthermore, the skilled person, without undue experimentation, would be able to demonstrate that a conjugate has the desired technical properties. In this regard, as discussed on pages 2-3 of the present specification, the basic

concept of re-targeting a toxin to a desired target cell was well documented prior to the present application.

The Office Action concedes on page 13 that "methods for making fusion proteins are well known in the art." The present application describes in the passage spanning pages 10-11 that the components of the conjugates are typically linked together (directly or via linker molecules) by covalent bonds, using conventional conjugation reagents/ techniques (see page 21, lines 3-6). In this regard, it would be a matter of routine for a skilled person to carry out native polyacrylamide gel electrophoresis to confirm that a conjugate of the present invention has the anticipated molecular weight.

Preparation of a conjugate according to the present invention is described in detail on pages 19-23 of the present specification. In this regard, where the targeting moiety / agonist and protease components are derived from a clostridial neurotoxin, these components may be obtained by removing or mutating / blocking / modifying the H_C portion of a clostridial neurotoxin to reduce or incapacitate the native binding activity of the clostridial neurotoxin.

Alternatively, the H_N portion of a clostridial neurotoxin may be covalently linked to a L-chain of a different clostridial or non-clostridial neurotoxin (or any other non-cytotoxic protease having the desired technical properties) and this hybrid is subsequently covalently linked to a targeting moiety / agonist. Where the targeting moiety / agonist activity and the translocation activity are provided by the same molecule, this molecule may be covalently linked to the non-cytotoxic protease.

Conjugates according to the present invention may also be prepared recombinantly, as described in detail on pages 21-23 of the present specification. In this regard, as discussed on page 21, lines 25-36, all the basic information required for recombinant production of the present

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS^{PLLC}
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

-20-

MASQ\27218AM2.DOC

conjugates was readily available prior to the present application, or was readily determinable by a skilled person using conventional methods.

Furthermore, a skilled person would consider it routine to detect binding of a conjugate to a binding site on a target cell (via the targeting moiety / agonist component), and subsequent receptor-mediated endocytosis, using a range of standard methodologies, such as those described on page 11, lines 6-28, of the present specification.

Examples 1-27 and Figure 3 of the present specification illustrate a range of methodologies for preparing conjugates using a number of different targeting moieties / agonists, translocation domains and proteases, and methodologies for assessing conjugate activity *in vitro* and *in vivo*.

In the light of the present specification and routine knowledge in the art at the time of the invention, a skilled person would consider it routine to prepare a non-cytotoxic toxin conjugate of the present invention, and would face no undue burden in doing so. Thus, the conjugates recited in the pending claims are fully enabled by the specification as filed, and withdrawal of the rejection is respectfully requested.

<u>The Rejection of Claims 58 and 60-75 Under 35 U.S.C. § 112, First Paragraph</u>

Claims 58 and 60-75 stand rejected under 35 U.S.C. § 112, first paragraph, for failing to describe claimed subject matter in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Office Action states the specification only teaches a single representative species to support the claims, which are directed to a genus of methods for making conjugates. Further, the Office Action states that the specification fails to describe representative species by any identifying characteristics or properties other than the functionality of being a method for making the claimed conjugates. Based on this, the Office Action concludes that the specification fails to

sufficiently describe the claimed invention in such full, clear and exact terms that a skilled artisan would recognize that applicants were in possession of the claimed invention. Applicants respectfully traverse the rejection for the following reasons.

As described above in detail, the present specification discloses conjugates with a range of targeting moiety, protease, and translocation domains beyond the IL-13 targeting agonist, catalytic, and targeting moiety domains described in Example 8 (see Examples 3, 11, 15, 19, 22, and 25). The structures of these fragments have been extensively characterized in the art, and thus, their expected tolerance to modification would be well-known to the skilled artisan. Therefore, applicants respectfully submit that these examples provide sufficient guidance and multiple examples such that a skilled artisan would recognize that applicants were in possession of the claimed invention.

Also as described above, based on the present specification it would be a matter of routine for a skilled artisan to identify alternative targeting moieties, translocation domains, and proteases to generate a conjugate according to the invention. Thus, the specification provides sufficient description such that a skilled artisan would recognize that applicants were in possession of the claimed invention.

Briefly, targeting moieties can be identified by conventional methods, including routine literature searches and assaying cellular secretion or concentration of a membrane protein in a target cell. The specification not only provides a discussion of representative techniques for these assays (page 6, lines 19-23 and page 6, line 25, to page 7, line 2, respectively), but also illustrates a number of them in the Examples (see, e.g., Examples 1, 9, 18, 23, and 26). The specification discloses that routine assays to confirm the effectiveness of the agonist molecules (see. e.g., page 7, lines 13-17, page 10, lines 7-20, and Examples 1, 9, 14, 18, 21, 23 and 27) and provides details of the assays on pages 10-11.

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS^PLLC
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

-22-

MASQ\27218AM2.DOC

Likewise, suitable proteases can be identified based on guidance provided by the specification and routine assays known in the art, evidencing possession of the invention. As described above, identification of a protease that is nontoxic and that cleaves a target protein is routine in the art (such as by measuring cell viability and the monitoring of substrate using an antibody, respectively). Furthermore, the specification provides numerous specific examples of proteases suitable for use in the claimed conjugates.

Likewise, suitable translocation domains can be identified based on guidance provided by the specification and routine assays known in the art, evidencing possession of the invention. As described above, well-known assays for determining whether a molecule possesses the require translocation activity are discussed on page 13, lines 5-25 of the present specification. Additionally, the specification provides discussion of well-known translocation activities of the H_N domains of clostridial neurotoxins at page 2, lines 1-9 and pages 13-14. The specification also provides examples of known non-clostridial translocation domains at pages 14-16, particularly Table 1.

Likewise, the assembly of the above-described components to generate a conjugate is discussed in the specification, evidencing possession of the invention. The specification describes on pages 2-3 that the basic concept of retargeting conjugates was well documented prior to the application. The specification discloses that routine conjugation techniques can be used in the claimed invention, in the passage spanning pages 10-11, and at page 21, lines 3-6. The specification describes the preparation of conjugates according to the invention at pages 21-23, including guidance regarding components derived from clostridial neurotoxins and production of recombinant conjugates. The specification describes standard methodologies to detect binding of conjugates to a binding site on a target cell and subsequent receptor-mediated endocytosis at page 11, lines 6-28. Furthermore, the specification provides in Examples 1-27,

MASQ07218AM2.DOC

and Figure 3 illustrations of a range of methodologies for preparing conjugates using a range of different targeting moieties, translocation domains, and proteases, and methodologies for assessing conjugate activity *in vitro* and *in vivo*.

Thus, applicants submit that the specification provides a description sufficient such that the skilled person would recognize that the inventors were in possession of the claimed invention as of the filing date of the application. Withdrawal of the rejection is respectfully requested.

The Rejection of Claims 58, 60, 62, 64, 74, and 75 Under 35 U.S.C. §§ 102(a)/(e)

Claims 58, 60, 62, 64, 74, and 75 stand rejected under 35 U.S.C. §§ 102(a)/(e) as being anticipated by U.S. Patent Application Publication No: 2003/0059912A1 ("Bigalke"). Withdrawal of the rejection is requested for the following reasons.

Claim 58 is an independent claim directed to a method of preparing a non-cytotoxic toxin conjugate for inhibition or reduction of exocytic fusion in a target cell. Claims 60, 62, 64, 74, and 75 depend from Claim 58.

The claimed method includes the steps of identifying an agonist that increases exocytic fusion in the target cell and preparing an agent. The agent includes the following components: (i) a targeting moiety that binds the agent to a binding site on the target cell, the binding site undergoing endocytosis to be incorporated into an endosome with the target cell, and wherein the targeting moiety is an agonist identifiable in the prior step; (ii) a non-cytotoxic protease or a fragment thereof, the protease or protease fragment being capable of cleaving a protein of the exocytic fusion apparatus of the target cell; and (iii) a translocation domain that translocates the protease or protease fragment from within the endosome, across the endosomal membrane, and into the cytosol of the target cell.

The pivotal step underlying the claimed method is the selection of a particular type of targeting moiety (TM) that is used to target the conjugate to the target cell. Importantly, the TM

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS*^PLLC*
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

-24-

MASQ\27218AM2.DOC

is an <u>agonist that increases exocytic fusion</u> in the target cell. The Bigalke reference fails to teach at least the element of identifying an <u>agonist</u> that increases exocytic fusion in the target cell, as recited in Claim 58.

The Bigalke reference is directed to a hybrid protein for inhibiting the degranulation of mastocytes and the use thereof. The hybrid proteins contain a protease for cleaving one or several proteins of the secretion process and a transporter protein that specifically binds to and are endocytosed by mastocytes and basophils. The transporter protein channels the protease into the appropriate cell, wherein the protease is able to specifically cleave proteins of the fusion complex, thus preventing degranulation.

In contrast to the claimed invention, the TMs used in the Bigalke reference are not agonists that increase exocytic fusion in the target cell. Indeed, the whole aim of the Bigalke reference is to <u>inhibit</u> exocytic fusion in the target cells (see, e.g., title "inhibiting the degranulation", abstract "inhibit the secretion process", paragraph [0032] "blocks the degranulation and the secretory mechanism").

The IgE (and related molecules) used as TMs in the cited reference do not induce exocytic fusion in the target cell. The presence of a <u>separate allergen</u> is required before exocytic fusion will occur. This is confirmed by col. 1, paragraph [0003] of D1, and also by the review paper by Puxeddu et al (2003) (see, e.g., page 1603, section 2.4), enclosed herewith as **Exhibit A**. The process of activation of the IgE receptor resulting in mast cell degranulation is a <u>two-step</u> process. The first step involves the binding of IgE to the IgE receptor. This process is known as sensitisation or priming and does not lead to mast cell degranulation. To achieve mast cell degranulation, the IgE receptor must be cross-linked by the binding of an allergen to allergen-specific IgE bound to the IgE receptor.

Thus, it is clear that the IgE (and related molecules) described in the Bigalke reference are not agonists that increase exocytic fusion in the target cell.

The Bigalke reference describes the use of a TM that includes an <u>inactive</u> Mast Cell Degranulating peptide (MCD peptide) (see, e.g., paragraphs [0016], [0033], Example 3). Again, this TM is not acting as an agonist that increases exocytic function in the target cell. The MCD peptide is <u>inactive</u> and cannot stimulate exocytic fusion. As noted above, the stimulation of exocytic fusion by the MCD peptide would be entirely against the teaching and aim of the cited reference, which is to <u>inhibit</u> the degranulation of mastocytes.

Because the cited reference fails to exactly describe the claimed invention, the reference is not anticipatory. Withdrawal of the rejection is respectfully requested.

<u>The Rejection of Claims 58 and 60-75 Under 35 U.S.C. § 103(a)</u>

Claims 58, 60-65, 67-69, and 72-75 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the Bigalke reference in view of Skeberdis et al., 2001 ("Skeberdis").

Claims 58, 60-65, 67, 70, 71, 74, and 75 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the Bigalke reference in view of Foran et al., 1999 ("Foran").

Claim 66 stands rejected under 35 U.S.C. § 103(a) as being unpatentable over the Bigalke reference in view of Yoshimaru et al., 2002 ("Yoshimaru").

Withdrawal of these rejections is requested for the following reasons.

As noted above, Claim 58 is an independent claim directed to a method of preparing a non-cytotoxic toxin conjugate for inhibition or reduction of exocytic fusion in a target cell. Claims 60, 62, 64, 74, and 75 depend from Claim 58.

The claimed method includes the steps of identifying an agonist that increases exocytic fusion in the target cell and preparing an agent. The agent includes a targeting moiety that binds the agent to a binding site on the target cell, the binding site undergoing endocytosis to be

MASQ\27218AM2.DOC

incorporated into an endosome with the target cell, and the targeting moiety is an agonist identifiable in the first step of the claimed method.

To establish a *prima facie* case of obviousness, the cited prior art references must teach or suggest all of the claim elements. In addition, there must be some apparent reason, either in the references or in the knowledge of one skilled in the art, to modify the reference or to combine the elements of multiple references with a reasonable expectation of success. Applicants respectfully submit that no reason or motivation exists within the references or knowledge existing in the art to modify or combine the cited references to arrive at the claimed method.

To the contrary, Bigalke teaches away from using an agonist as part of a toxin conjugate for inhibition or reduction of exocytic fusion in a target cell, as recited in Claim 58.

A problem addressed by the present invention is the provision of methods for preparing re-targeted toxin conjugates for inhibiting or reducing exocytic fusion in a target cell. This problem is solved by the method of Claims 58 and 60-75.

An inventive step underlying the present invention rests in the selection of a particular type of targeting moiety (TM) that is used to target the conjugate to the target cell. Specifically, the TM is an <u>agonist that increases exocytic fusion</u> in the target cell.

Applicants have unexpectedly found that taking this entirely counterintuitive step (i.e., targeting the conjugate using a TM that <u>stimulates</u> the very process that the conjugate is intended to inhibit) has a number of surprising advantages. By way of example, the present Inventors have identified that the use of an agonist TM can confer preferential binding and/or internalisation properties on a conjugate of the present invention, which may result in more efficient delivery of the non-cytotoxic protease component to the target cell. In addition, the use of an agonist as a TM can be self-limiting with respect to side-effects (see page 8). Applicants respectfully submit that neither the references nor the general knowledge within the art provide

MASQ27218AM2.DOC

any guidance to make this counterintuitive step of utilizing an agonist to prepare conjugate for inhibition or reduction of exocytic fusion in a cell.

Applicants submit that the skilled person, faced with the problem of choosing a TM for a conjugate that is intended to inhibit/reduce exocytic fusion in a cell, would not consider it obvious to use a TM that has <u>precisely the opposite effect</u> and actually stimulates exocytic fusion in the cell.

Thus, an inventive technical feature of the claimed method is the use of an agonist TM that increases exocytic fusion in a target cell. Selection of an appropriate TM involves consideration of the particular target cell, as the agonist properties of a TM cannot be assessed in isolation from the target cell. This technical feature is therefore very different from the methods described in, for example, the Bigalke reference, which relate to conventional re-targeting technology, and which do not teach or suggest any <u>additional</u> requirement for the TM to be an <u>agonist</u> of the target cell.

Thus, the pending claims are nonobvious in view of the Bigalke reference.

The deficiencies noted immediately above, and in response to the novelty rejection based on the Bigalke reference, are not cured by the teachings of the Skeberdis, Foran, or Yoshimaru references.

The Skeberdis reference teaches that insulin promotes rapid delivery of NMDA receptor channels to the cell surface by exocytosis. The Office Action cites Skeberdis as allegedly teaching that this exocytosis is inhibited by a clostridium toxin. The Office action asserts that it would have been obvious to a person of ordinary skill in the art to modify the method of Bigalke to prepare a conjugate comprising the targeting agonist insulin linked to a domain comprising the catalytic and translocation domains of a clostridial neurotoxin, wherein exocytosis of the NMDA receptor channel is detected by assaying the expression of the receptor/channel at the plasma

MASQ27218AM2.DOC

membrane, as taught by Skeberdis. The Office Action states that motivation to combine is derived from the desire to develop an agent useful for modulating the number of synaptic NMDA receptors channels, which are involved in memory and learning processes. Applicants respectfully disagree. Skeberdis' use of the light chain of type A botulinum neurotoxin is merely as an assay to establish that insulin increases the number of NMDA channels at the cell surface by causing insertion of new channels instead of unmasking of silent channels (Skeberdis, p3564, right column; p3565, right column). Skeberdis provides absolutely no suggestion that insulin should be used, or is even capable of being used, as part of a conjugate to inhibit or reduce the exocytic process that it stimulates.

The Foran reference teaches that a protein kinase B and insulin stimulate the translocation of various glucose transporters and transferrin receptors to the plasma membrane. The Office Action cites Foran as teaching that insulin increases plasma membrane expression of GLUT4 transporter via exocytosis and this exocytosis is inhibited by a clostridium toxin. The Office Action asserts that it would have been obvious to a person of ordinary skill in the art to modify the method of Bigalke to prepare an agent comprising the targeting agonist insulin linked to a domain comprising the catalytic and translocation domains of a clostridial neurotoxin, wherein exocytosis of the GLUT4 transporter is detected by assaying the expression of the transporter at the plasma membrane as taught by Foran. The Office Action states that motivation to do so is derived from the desire to develop an agent useful for modulating the number of plasma membrane GLUT4 transporters, which is involved in glucose uptake. Applicants respectfully disagree. As in Skeberdis, Foran's use of clostridium toxins (here, the light chains of botulinum toxin types A, B, C1 and E) is merely as an assay tool to establish whether insulin (or a constitutively active protein kinase B) stimulated translocation of various membrane proteins via SNARE-protein trafficking mechanisms or SNARE-independent trafficking pathways. Foran

provides absolutely no suggestion that insulin or protein kinase B should be used, or is even capable of being used, as part of a conjugate to inhibit or reduce the exocytic process that they stimulate.

The Skeberdis and Foran references merely describe isolated molecules that are allegedly capable of stimulating receptor expression (via exocytosis) in certain types of cell. These references provide absolutely no suggestion to a skilled person that these molecules should - or even could - be used for the re-targeting of toxin conjugates. Furthermore, as discussed above, a skilled person would not consider the molecules described in the Skeberdis and Foran references to be suitable as TMs for re-targeting toxin conjugates for <u>inhibiting or reducing</u> exocytic fusion, due to their <u>stimulatory</u> effect on secretion from a target cell.

The Yoshimaru reference teaches that the blockade of superoxide generation prevents high-affinity IgE receptor-mediated release of allergic mediators in rat mast cells and human basophils. The Office Action asserts that it would have been obvious to a person of ordinary skill in the art to modify the method of Bigalke to prepare an agent comprising the targeting agonist IgE linked to a domain comprising the catalytic and translocation domains of a clostridial neurotoxin, wherein exocytosis of histamine is detected by ELISA. Applicants respectfully disagree. The Yoshimaru reference simply describes the use of ELISA to detect the release of histamine from mast cells after stimulation using IgE and specific antigen. This reference provides no teaching whatsoever towards using an agonist as a targeting moiety to inhibit or reduce exocytic fusion in target cells. This reference provides no teaching whatsoever towards re-targeting toxin conjugates using agonist TMs.

Therefore, the identification that an agonist molecule - which acts to <u>increase</u> exocytic fusion in a target cell – can be used for re-targeting a non-cytotoxic protease to a target cell in

order to <u>reduce/ inhibit</u> exocytic fusion from that target cell, a recited step of Claim 58, is neither taught nor suggested in the cited references.

Because the cited references, either alone or in any combination, fail to teach, suggest, provide any motivation to make, or otherwise render obvious the method as now claimed, the claimed method is nonobvious and patentable over the cited references. Withdrawal of the rejections are respectfully requested.

<div align="center">CONCLUSION</div>

In view of the above amendments and foregoing remarks, applicants believe that Claims 58 and 60-75 are in condition for allowance. If any issues remain that may be expeditiously addressed in a telephone interview, the Examiner is encouraged to telephone applicants' attorney at 206.695.1755.

Respectfully submitted,

CHRISTENSEN O'CONNOR
JOHNSON KINDNESS[PLLC]

George E. Renzoni

George E. Renzoni, Ph.D.
Registration No. 37,919
Direct Dial No. 206.695.1755

GER:tsn

LAW OFFICES OF
CHRISTENSEN O'CONNOR JOHNSON KINDNESS[PLLC]
1420 Fifth Avenue
Suite 2800
Seattle, Washington 98101
206.682.8100

-31-

MASQ\27218AM2.DOC

EXHIBIT A

IJBCB

Cells in focus

Mast cells in allergy and beyond

I. Puxeddu, A.M. Piliponsky, I. Bachelet, F. Levi-Schaffer*

*Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem,
P.O. Box 12065, 91120 Jerusalem, Israel*

Abstract

Mast cells (MC) are highly granulated tissue dwelling cells, widely distributed throughout the body in connective tissues and on mucosal surfaces. They are derived from bone marrow progenitors that migrate into the blood and subsequently into the tissues, where they undergo final maturation. Mast cell proliferation, differentiation, survival and activation are regulated by stem cell factor, the ligand for the c-kit tyrosine kinase receptor, expressed on the mast cell surface. They release a large number of pro-inflammatory and immunoregulatory mediators after activation induced by either immunoglobulin E-dependent or immunoglobulin E-independent mechanisms. Mast cells have been most widely studied in the context of allergic reactions and parasite infections, but there is now compelling evidences that they are important players in innate and acquired immunity, wound healing, fibrosis, tumors and autoimmune diseases. This review will discuss current advances in these fields.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: Mast cells; c-kit; Stem cell factor; Allergy

Cell facts

- Mast cells are high affinity IgE receptor bearing tissue dwelling cells containing prominent cytoplasmic granules and key cells in allergy.
- Mast cell proliferation, differentiation, survival and activation are regulated by stem cell factor.
- Mast cells and their mediators participate in innate and acquired immunity, wound healing, tissue remodeling, angiogenesis and autoimmune diseases.

---

1. Introduction

Mast cells (MC) are tissue dwelling cells containing prominent cytoplasmic granules. They have a pivotal role in allergic reactions and take part in other pathophysiological conditions such as innate and acquired immunity (Mekori & Metcalfe, 2000), wound healing, fibrosis, tumors and autoimmune diseases (Benoist &

Mathis, 2002) (Fig. 1). MC are widely distributed throughout the body, in connective tissues and on mucosal surfaces where they are frequently located in close proximity to blood vessels and peripheral nerves. Due to this strategic location, they are exposed to environmental stimuli such as microorganisms and allergens (Galli, Maurer, & Lantz, 1999) with which they can react, both within minutes and/or over a period of hours, and undergo regulated secretion of preformed and newly synthesized mediators. Until very recently, studies on human MC have been hampered because of the difficulty to obtain large amounts of highly

* Corresponding author. Tel.: +972-2-6757512;
fax: +972-2-6758144.
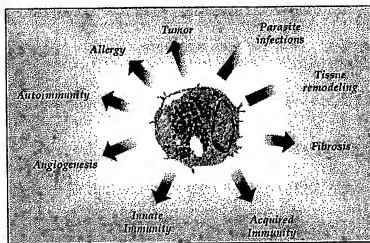*E-mail address:* fls@cc.huji.ac.il (F. Levi-Schaffer).

Fig. 1. Roles of mast cells in different physiopathological processes. Mast cell versatility is demonstrated by its participation in many different pathophysiological processes.

purified MC. Recently, however, with new methodologies for human MC obtention, many advances have been made in the understanding of MC biology.

This article provides new evidences on the well-established properties of MC in type I hypersensitivity reactions and on other less "classical" pathophysiological roles.

2. Cell origin and plasticity

2.1. Mast cell origin and development

MC are derived from progenitors expressing CD34, CD117 (c-kit) and CD13 in humans, and from Thy-1^{lo} c-kit^{hi} cells in the mouse, circulating in the peripheral blood and lymphatics and migrating into vascularized tissues, where they undergo final maturation under the influence of local factors. Several studies indicate that MC proliferation, differentiation and survival are stringently regulated by stem cell factor (SCF), the ligand for the c-kit tyrosine kinase growth factor receptor, which is expressed on the MC surface. In addition, other mediators such as interleukin (IL)-3, IL-4, IL-9, IL-10, nerve growth factor (NGF), some chemokines and retinoids can regulate MC differentiation (Marone, Galli, & Kitamura, 2002). Before full granule maturation, circulating progenitors of MC also display the low affinity IgG receptor FcγRIIb, and contain mRNA

for MC proteases. MC express the high affinity IgE receptor, FcϵRI, early in their development, but apparently only after they undergo lineage-commitment.

2.2. Mast cell survival and death

MC numbers in tissues are relatively constant, even though MC hyperplasia is observed in both the inflammatory and in the repair/remodeling stage of various inflammatory/fibrotic disorders (Bischoff & Sellge, 2002).

The survival of human mature MC in tissues appears to depend largely on the local production of SCF since its withdrawal results in apoptosis (Iemura, Tsai, Ando, Wershil, & Galli, 1994). Moreover, other cytokines are involved in MC survival and growth. For example, IL-3 induces mouse MC growth and enhances their development in response to SCF in vitro while in humans its role is still controversial. MC progenitors and human intestinal MC (Bischoff & Sellge, 2002) but not tissue-derived mature MC from lung, uterus, kidney, tonsils and skin express IL-3 receptor. NGF has also been reported to be a survival factor for rat peritoneal MC (RPMC) (Kawamoto et al., 1995) and for human cord blood-derived mast cells (CBMC) in the presence of SCF (Kanbe et al., 2000).

Mouse bone marrow derived-MC (BMMC), RPMC and the murine C57 and MCP-5 lines express the death receptor Fas (Apo-1/CD95), but only the C57

are susceptible to Fas dependent apoptosis. We have recently found that human lung and CBMC express functional tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) receptors and that SCF prevents their apoptosis induced by TRAIL receptor ligation. This would suggest a novel mechanism for the pro-survival effects of SCF on MC.

2.3. Mast cell heterogeneity

MC can be heterogeneous in phenotype and functions (Metcalfe, Baram, & Mekori, 1997). Historically, in rodents two main MC subtypes have been described according to their phenotype and tissue localization: (i) T cell-dependent mucosal MC (MMC), mainly found in the mucosa of the gastrointestinal system and in the lamina propria of the respiratory tract; (ii) T cell-independent connective tissue MC (CTMC) localized in the sub-mucosa of the gastrointestinal tract, in the skin and in the peritoneum. However, the MMC and CTMC differ in many other aspects. In humans, by analogy to rodents, two main MC subtypes have been described, according to their distinct protease patterns: one residing preferentially in mucosal tissues and containing only tryptase (MC$_T$); the other one found in connective tissues containing tryptase, chymase, cathepsin G and carboxypeptidase (MC$_{TC}$) (Table 1). In addition MC that express only chymase (MC$_C$) have been shown in several human tissues (Weidner & Austen, 1993) and in bone marrow during myelodysplasia or mastocytosis (Horny, Greschniok,

Jordan, Menke, & Valent, 2003), although their role has not yet been elucidated. Human MC phenotypes are also heterogenic in their cytokine content. For example, IL-4 is distributed preferentially in the MC$_{TC}$ subset. In contrast, IL-5 and IL-6 is restricted almost exclusively to the MC$_T$ subset. In addition to these differences, both MC$_T$ and MC$_{TC}$ express FcεRI and participate in IgE-dependent allergic or anti-parasite reactions. It is important to note that CBMC and the human mast cell line HMC-1, derived from a patient with leukemia, frequently used as a source of MC for in vitro studies, are mainly MC$_T$.

2.4. Mast cell activation

MC undergo regulated exocytosis when a multivalent antigen causes the aggregation of occupied FcεRI on their surface. This event is followed by the immediate release of preformed granular mediators such as histamine, tryptase and chymase (much more slowly). Activated MC can also very rapidly synthesize and release prostaglandin (PG)-D$_2$, thromboxanes, leukotrienes (LTs) and platelet-activating factor (PAF). MC are important sources of an array of cytokines, growth factors, and chemokines (Mekori & Metcalfe, 2000).

CBMC are activated by IgG-dependent stimuli only after incubation with interferon (IFN)-γ that upregulates the expression of FcγRI. Under these conditions the expression of FcγRII is also increased, but it does not induce MC activation. In contrast, the expression

Table I
Characteristics of the two major human mast cell phenotypes

Classification	MC$_T$ (immune system associated)	MC$_{TC}$ (non-immune system associated)
Location	GI tract mucosa, respiratory tract, lamina propria	Sub-mucosa of GI tract and skin
IgE dependency	IgE-dependent activation IgE-independent activation[a]	IgE-dependent and independent activation
Major enzymes	Tryptase	Tryptase, chymase, cathepsin G, carboxypeptidase
Major arachidonic acid metabolites	LTC$_4$, PG-D$_2$ (↓)	LTC$_4$ (↓), PG-D$_2$
Major cytokines	IL-5 (↓), IL-6, IL-7, IL-8, IL-10, IL-13, IL-16, TNF-α, GM-CSF, SCF, TGF-β	IL-5 (↓), IL-6 (↓), IL-3, IL-4, IL-7, IL-8, IL-10, IL-13, IL-16, TNF-α, SCF
Pathological phenotype	Increased around sites of T$_H$-activation Increased in allergic and parasitic diseases Decreased in AIDS and chronic immunodeficiency diseases	Increased in fibrotic diseases Unchanged in allergic and parasitic diseases Unchanged in AIDS and chronic immunodeficiency diseases

MC$_T$: mast cells containing only tryptase; MC$_{TC}$: mast cells containing both tryptase and chymase; (↓): decrease.
  [a] Recently shown by our group (Piliponsky et al., 2003).

of FcγRIII is not increased (Woolhiser, Okayama, Gilfillan, & Metcalfe, 2001). RPMC and MCTC isolated from the skin degranulate in response to several non-immunological agents, such as compound 48/80, basic polypeptides (polylysine, polyarginine), morphine sulphate, substance P, and the anaphylatoxin C5a (Lowman, Rees, Benyon, & Church, 1988). In addition compound 48/80 tested in the skin of patients with scleroderma increased local MC releasability compared with healthy subjects, suggesting that MC in disease can differ in their functional properties than MC in health (Pearson et al., 1988). In contrast to RPMC and MCTC, murine BMMC and MCT have been described as unresponsive to IgE-independent activation. However, MC activation can be profoundly affected by the microenvironment. For example, mouse BMMC were shown to acquire responsiveness to IgE-independent stimuli when in co-culture with mouse 3T3 fibroblasts. In accordance with this, we have recently observed that human lung mast cells (HLMC), that are mostly MCT, become responsive to eosinophil major basic protein (MBP) when in co-culture with human lung fibroblasts and that the fibroblast-derived membrane form of SCF is responsible for this process (Piliponsky, Gleich, Nagler, Bar, & Levi-Schaffer, 2003). MC also express a large number of surface receptors (Ott & Cambier, 2000), that inhibit the release of pro-inflammatory mediators. For example Katz et al. have recently demonstrated in gp49B−/− mice that activation of the Ig superfamily member gp49B1 on MC inhibits their activation upon IgE-dependent and independent stimuli, suggesting new approaches to modulate the allergic reactions.

3. Cell function and associated pathology

3.1. Mast cells in allergic inflammation

In allergy, apart from their classical role in eliciting the early phase, MC also have an important function in its late and chronic stages. In these stages they may interact with and be activated by infiltrated inflammatory cells such as eosinophils and lymphocytes and by resident structural cells such as epithelial, smooth muscle cells and fibroblasts (Fig. 2). Most of the evidences for these interactions come from in vitro studies. For example, we have found that IgE-challenged RPMC
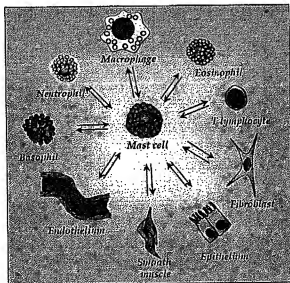

Fig. 2. Mast cell interactions with other cells during allergic inflammation. The interactions between the mast cell and several types of cells are explained in further detail in the text.

are sensitive to reactivation induced by eosinophil sonicate and eosinophil-derived MBP (Piliponsky, Pickholtz, Gleich, & Levi-Schaffer, 2001). In addition, HLMC are activated by MBP to release histamine and PG-D2 when in co-culture with human lung fibroblasts (Piliponsky et al., 2003). Activated MC release mediators that contribute to eosinophil recruitment, activation and survival. For example, MC-derived TNF-α enhances eosinophil survival through autocrine production of granulocyte–macrophage colony stimulating factor (GM-CSF), a process regulated by nuclear factor kappa B (NFκB) and sensitive to steroids (Levi-Schaffer, Temkin, Malamud, Feld, & Zilberman, 1998). In addition, MC-derived tryptase induces IL-6 and IL-8 release in eosinophils by the mitogen-activated protein kinase (MAPK)/activator protein (AP)-1 pathway (Temkin, Kantor, Weg, Hartman, & Levi-Schaffer, 2002). MC also stimulate macrophages and epithelial cells to generate lymphocyte and eosinophil chemoattractants, and through the release of IL-4, they promote the development of Th2. In asthma it has been recently observed that the number of infiltrated MC into airway smooth muscle correlates with the degree of airway hyperresponsiveness, a phenomena attributed in the past exclusively to the eosinophils (Brightling et al., 2002).

3.2. Mast cells in innate immunity

Since MC are mainly found in the host–environment interface from which microorganisms can penetrate, they can constitute one of the first inflammatory cells establishing a direct contact with the invading pathogens. Using MC c-kit deficient W/W^v mice and the c-kit knock-in mice it was demonstrated that during a bacterial infection induced by lipopolysaccharide (LPS), MC specifically secrete TNF-α to recruit into the site of infection circulating leukocytes with bactericidal properties such as neutrophils (Malaviya, Ikeda, Ross, & Abraham, 1996). In addition, Maurer et al. found that repetitive administration of SCF and consequent MC hyperplasia, can improve the survival of normal mice with acute bacterial peritonitis induced by cecal ligation and puncture (CLP) (Maurer et al., 1998). In these cases MC can be triggered by the interaction of CD48, a glycosylphosphatidylinositol-anchored molecule expressed in MC, or by Toll-like receptor (TLR)-2. For example, bacterial peptidoglycan and yeast zymosan were potent inducers of GM-CSF, IL-1β, and LTs generation in TLR-2 activated CBMC while a synthetic triacylated lipopeptide induced short-term degranulation, but failed to induce LTs production (McCurdy, Olynych, Maher, & Marshall, 2003). During bacterial infection MC can also be activated by the host's complement system, since in vivo inflammatory response to CLP is significantly reduced in C4- or C3-deficient mice compared to wild-type mice.

New evidences indicate that MC are also involved in HIV-1 infection. The HIV-1 glycoprotein gp120 stimulates IL-4 and IL-13 release from MC via gp120 interaction with the V$_H$3 region of IgE. In addition, the Tat protein, secreted by HIV-1 infected cells, is a potent chemoattractant for FcεRI$^+$ cells and upregulates the expression of CCR3 on their surface.

3.3. Mast cells in acquired immunity

The role of MC in acquired immunity has been demonstrated to be regulated by IL-3 which is probably derived from T cells that recognize the infecting agents. IL-3 enhances immunity in mice infected with the intestinal nematode *Stronglyoides venezuelensis* and increases the number of tissue MC. In addition, IL-4 and IL-13 are required for the MC response that induces the expulsion of *Trichinella spiralis* and *Trichuris muris*.

The role of MC in acquired immunity as antigen presenting cells (APC) has also been suggested. In fact human MC constitutively express MHC class I molecules and express MHC class II molecules after treatment with IFN-γ and TNF-α. Furthermore MHC class II molecules are found in HMC-1 and find the staphylococcal enterotoxin A (SEA) leading to ultrastructural changes that suggest MC degranulation.

3.4. Mast cells in tissue remodeling, fibrosis and angiogenesis

MC have been implicated in physiological wound healing as well as in fibrosis. In several fibrotic diseases such idiopathic lung fibrosis, bleomycin or radiation-induced fibrosis, asthma, scleroderma, chronic graft-versus-host-disease (cGvHD), liver fibrosis, Crohn's disease and inflammatory bowel disease (Levi-Schaffer & Piliponsky, 2003), MC hyperplasia and signs of degranulation are present. In a rodent model of peritoneal adhesions and of Crohn's disease nedocromil sodium, a MC stabilizer, decreased the severity of the disease (Xu et al., 2002), indicating the important pathogenic role of the MC in chronic inflammation and fibrosis.

MC have the potential to directly influence the fibroblasts, the main players of fibrosis, and/or indirectly other cells leading to a pro-fibrotic response. For example, HMC-1, that produces matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, induces human lung, skin and intestinal fibroblasts proliferation by mediators such as histamine and tryptase and enables retraction of the collagen matrix. It is interesting to note that while HMC-1 increases collagen production in the skin (Garbuzenko et al., 2002), it decreases collagen production in the lung and intestine (Xu, Rivkind, Pappo, Pikarsky, & Levi-Schaffer, 2002). Some of the MC associated cytokines such as transforming growth factor (TGF)-β, IL-4, IL-6, IL-13, and NGF display pro-fibrogenic activities. For example, NGF enhances human lung, skin and conjunctival fibroblasts migration, differentiation into myofibroblasts, and contraction of collagen gels, but not proliferation and collagen synthesis, indicating

an important role of this factor at the beginning and end stages of wound repair (Micera et al., 2001).

MC have also been linked for almost two decades to neovascularization, during haemangioma, rheumatoid arthritis (RA), nasal polyps, wound healing and ovulation (Levi-Schaffer & Pe'er, 2001). Human inflamed tissue and murine MC from tissue with anaphylaxis were shown to contain in their cytoplasmic granules fibroblast growth factor (FGF)-2 and vascular endothelial growth factor (VEGF). In addition heparin, histamine, tryptase, TGF-β, TNF-α, IL-8 have been shown to have pro-angiogenic properties, while others such as endostatin have anti-angiogenic effects.

3.5. Mast cells in autoimmune diseases

MC have been implicated in several autoimmune diseases, including Sjogren's syndrome, chronic idiopathic urticaria, autoimmune thyroiditis and experimental vasculitis. A correlation between the number and/or distribution of mast cells and the development of experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis, has been reported (Benoist & Mathis, 2002). The direct contribution of MC in EAE comes from studies in mast cell deficient W/W^v mice, where the onset of myelin oligodendrocyte glycoproteins (MOG)-induced EAE is delayed and less severe than in control mice. MC might also have an early and coordinating role in the pathogenesis of RA. An increased number of MC and tryptase are found in the swollen paws of mice with collagen-induced arthritis and in the synovial fluid of patients with RA (Benoist & Mathis, 2002). In this context, the MC-derived TNF-α may stimulate the synovial fibroblasts to produce SCF, suggesting a potential mechanism for the perpetuation and amplification of the local inflammation in this pathological condition.

4. Conclusions

As reported in this review, during the last few years novel aspects of MC biology have been discovered. Based on these findings new frontiers have opened to explore and understand the complex role of these cells in health and disease. This may ultimately lead to novel approaches for the treatment and/or prophylaxis of mast cell-associated diseases.

References

Benoist, C., & Mathis, D. (2002). Mast cells in autoimmune disease. *Nature, 420,* 875–878.

Bischoff, S. C., & Sellge, G. (2002). Mast cell hyperplasia: Role of cytokines. *International Archives of Allergy and Immunology, 127,* 118–122.

Brightling, C. E., Bradding, P., Symon, F. A., Holgate, S. T., Wardlaw, A. J., & Pavord, I. D. (2002). Mast cell infiltration of airway smooth muscle in asthma. *New England Journal of Medicine, 346,* 1699–1705.

Galli, S. J., Maurer, M., & Lantz, C. S. (1999). Mast cells as sentinels of innate immunity. *Current Opinion in Immunology, 11,* 53–59.

Garbuzenko, E., Nagler, A., Pickholtz, D., Gillery, P., Reich, R., Maquart, F. X., & Levi-Schaffer, F. (2002). Human mast cells stimulate fibroblast proliferation, collagen synthesis and lattice contraction: A direct role for mast cells in skin fibrosis. *Clinical and Experimental Allergy, 32,* 237–246.

Horny, H. P., Greschniok, A., Jordan, J. H., Menke, D. M., & Valent, P. (2003). Chymase expressing bone marrow mast cells in mastocytosis and myelodysplastic syndromes: An immunohistochemical and morphometric study. *Journal of Clinical Pathology, 56,* 103–106.

Iemura, A., Tsai, M., Ando, A., Wershil, B. K., & Galli, S. J. (1994). The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *American Journal of Pathology, 144,* 321–328.

Kanbe, N., Kurosawa, M., Miyachi, Y., Kanbe, M., Saitoh, H., & Matsuda, H. (2000). Nerve growth factor prevents apoptosis of cord blood-derived human cultured mast cells synergistically with stem cell factor. *Clinical and Experimental Allergy, 30,* 1113–1120.

Kawamoto, K., Okada, T., Kannan, Y., Ushio, H., Matsumoto, M., & Matsuda, H. (1995). Nerve growth factor prevents apoptosis of rat peritoneal mast cells through the *trk* proto-oncogene receptor. *Blood, 86,* 4638–4644.

Levi-Schaffer, F., & Pe'er, J. (2001). Mast cells and angiogenesis. *Clinical and Experimental Allergy, 31,* 521–524.

Levi-Schaffer, F., & Piliponsky, A. M. (2003). Tryptase, a novel link between allergic inflammation and fibrosis. *Trends in Immunology, 24,* 158–161.

Levi-Schaffer, F., Temkin, V., Malamud, V., Feld, S., & Zilberman, Y. (1998). Mast cells enhance eosinophil survival in vitro: Role of TNF-α and granulocyte–macrophage colony-stimulating factor. *Journal of Immunology, 160,* 5554–5562.

Lowman, M. A., Rees, P. H., Benyon, R. C., & Church, M. K. (1988). Human mast cell heterogeneity: Histamine release from mast cells dispersed from skin, lung, adenoids, tonsils and colon in response to IgE-dependent and nonimmunologic stimuli. *Journal of Allergy and Clinical Immunology, 81,* 590–597.

Malaviya, R., Ikeda, T., Ross, E., & Abraham, S. N. (1996). Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature, 381,* 77–80.

Marone, G., Galli, S. J., & Kitamura, Y. (2002). Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. *Trends in Immunology, 23,* 425–427.

Maurer, M., Echtenacher, B., Hultner, L., Kollias, G., Manuel, D. N., Langley, K. E., & Galli, S. J. (1998). The c-kit ligand, stem cell factor, can enhance innate immunity through effects on mast cells. *Journal of Experimental Medicine, 188,* 2343–2348.

McCurdy, J. D., Olynych, T. J., Maher, L. H., & Marshall, J. S. (2003). Distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *Journal of Immunology, 170,* 1625–1629.

Mekori, Y. A., & Metcalfe, D. D. (2000). Mast cells in innate immunity. *Immunological Reviews, 173,* 131–140.

Metcalfe, D. D., Baram, D., & Mekori, Y. A. (1997). Mast cells. *Physiological Reviews, 77,* 1033–1079.

Micera, A., Vigneti, E., Pickholtz, D., Reich, R., Pappo, O., Bonini, S., Maquart, F. X., Aloe, L., & Levi-Schaffer, F. (2001). Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair. *Proceedings of the National Academy of Sciences of the United States of America, 98,* 6162–6167.

Ott, V. L., & Cambier, J. C. (2000). Activating and inhibitory signaling in mast cells: New opportunities for therapeutic intervention? *Journal of Allergy and Clinical Immunology, 106,* 429–440.

Pearson, M. E., Huff, J. C., Giorno, R. C., Panicheewa, S., Claman, H. N., & Steigerwald, J. C. (1988). Immunologic dysfunction in scleroderma: Evidence for increased mast cell releasability and HLA-DR positivity in dermis. *Arthritis and Rheumatism, 31,* 672–677.

Piliponsky, A. M., Gleich, G. J., Nagler, A., Bar, I., & Levi-Schaffer, F. (2003). Non-IgE dependent activation of human lung and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood, 101,* 1898–1904.

Piliponsky, A. M., Pickholtz, D., Gleich, G. J., & Levi-Schaffer, F. (2001). Human eosinophils induce histamine release from antigen-activated rat peritoneal mast cells: A possible role for mast cells in late-phase allergic reactions. *Journal of Allergy and Clinical Immunology, 107,* 993–1000.

Temkin, V., Kantor, B., Weg, V., Hartman, M. L., & Levi-Schaffer, F. (2002). Tryptase activates the mitogen-activated protein kinase/activator protein-1 pathway in human peripheral blood eosinophils, causing cytokine production and release. *Journal of Immunology, 169,* 2662–2669.

Weidner, N., & Austen, K. F. (1993). Heterogeneity of mast cells at multiple body sites: Fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase and carboxypeptidase content. *Pathology, Research and Practice, 189,* 156–162.

Woolhiser, M. R., Okayama, Y., Gilfillan, A. M., & Metcalfe, D. D. (2001). IgG-dependent activation of human mast cells following up-regulation of FcgammaRI by INF-gamma. *European Journal of Immunology, 31,* 3298–3307.

Xu, X., Rivkind, A., Pappo, O., Pikarsky, A., & Levi-Schaffer, F. (2002). Role of mast cells and myofibroblasts in human peritoneal adhesion formation. *Annals of Surgery, 236,* 593–601.

Xu, X., Weksler-Zangen, S., Pikarsky, A., Pappo, O., Wengrower, D., Bischoff, S. C., Pines, M., Rivkind, A., Goldin, E., & Levi-Schaffer, F. (2002). Mast cells involvement in the inflammation and fibrosis development of TNBS-induced rat model of colitis. *Scandinavian Journal of Gastroenterology, 37,* 330–337.